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Treatment of chloroquine-resistant malaria with esters of cephalotaxine: homoharringtonine

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The esters of cephalotaxine–harringtonine, homoharringtonine and deoxyharringtonine—have been reported by both Chinese and American oncologists as useful in the treatment of human non-lymphoblastic leukaemias and selected solid tumours of the head and neck. We report our results with homoharringtonine, currently a Phase II clinical trial drug with the National Cancer Institute, in the treatment of malaria. Homoharringtonine, 2·7–3·4 nM, was effective in causing 50% growth inhibition of two strains of chloroquine-resistant *Plasmodium falciparum* malaria *in vitro*. *In vivo* tests in mice infected with *P. yoelii* showed that this drug was effective in inhibiting parasite growth in this system as well. Histologically, the drug was associated with karyorrhexis. Drug-exposed cells showed decreased levels of putrescine and spermidine and increased spermine levels. Our findings not only demonstrate the potential usefulness of homoharringtonine in the treatment of chloroquine-resistant malaria, but also demonstrate the advantage of applying comparative biochemistry and an understanding of biological mechanisms in a rational approach to the development and treatment of diseases including malaria.

Malaria has become an increasing problem in the tropical zones with the emergence of chloroquine-resistant strains and the decreased effectiveness of insecticides such as DDT. The magnitude of the problem is underlined by the fact that malaria is the most common infectious disease in the world. One billion people reside in endemic areas, and of these approximately 125–200 million people are diseased at any given time. In Africa alone over 1 million children die each year from malaria.

The problem is growing more serious as more strains of malaria are becoming more resistant to the major antimalarial drug, chloroquine. The major human pathogen is *Plasmodium falciparum*. More chloroquine-resistant strains of *P. falciparum* are emerging in Central and South America, Africa and Southeast Asia.

In an effort to combat new resistant malaria strains, chemists have synthesized chemical analogues of chloroquine. However, many of these strains already have become resistant to the new drugs. New drugs which have chemical properties totally different from chloroquine are needed to treat patients with these strains.

The authors noticed, while studying drug resistance and malaria-infected red cells and doing comparative biochemical analyses, that there was some similarity of these cells to tumour cells. Both tumour cells and parasite-infected cells exhibit exponential growth rates independent of the host. Some of the drugs available for treatment of diseases of these types may be considered to inhibit DNA or protein synthesis. We hypothesized that there may be similar mechanisms for defence or survival in different species. Drug resistance in both cases might have some common mechanisms (unpubl. obs.). We conjectured that there are only a certain number of mechanisms that organisms can use to offset or reverse potential noxious agents. We began testing antitumour agents for their efficacy as antimalarials. We were interested in cephalotaxine esters, especially harringtonine and homoharringtonine, because they are promising antitumour agents and have been found safe in human trials (Hsu, 1980;

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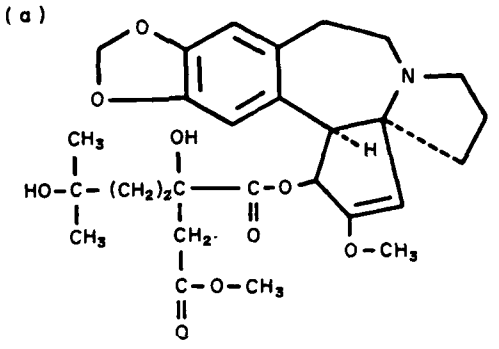
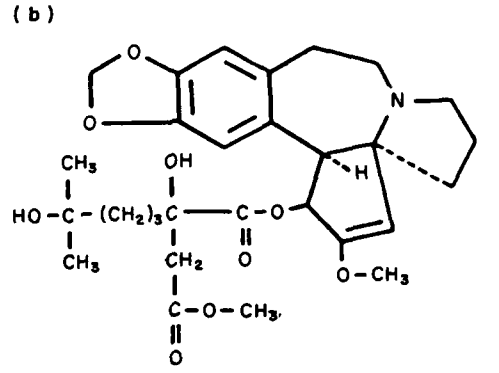
Hematology Research Division, 1980; Coonley *et al.*, 1983; Ohnuma and Holland, 1985; Warrell *et al.*, 1985; O'Dwyer *et al.*, 1986).

Homoharringtonine is derived from a well-known Chinese herb used for many years in Fujian, China, as a folk medicine for cancer. It is one group of cephalotaxine ester alkaloids which may be isolated from several species of *Cephalotaxus*, yew-like evergreen coniferous trees widely distributed in southern and northeastern China. Paudler *et al.* (1963) isolated several active principles from *Cephalotaxus drupacea* and *C. fortunei*. Powell *et al.* (1970), of the U.S. Department of Agriculture at Peoria, Illinois, isolated several alkaloids from *C. harringtonia* and defined the structure of homoharringtonine and harringtonine (Fig. 1). Homoharringtonine, a white powder insoluble in water but soluble in alcohol, has a large heterocyclic molecule with a molecular weight of 545·6. Harringtonine and homoharringtonine have been tested in human subjects by several clinical cancer groups, with the results summarized in a National Cancer Institute Clinical Brochure (1982) report.



Harringtonine



Homoharringtonine

Fig. 1. Structure of (a) harringtonine and (b) homoharringtonine.

The brochure includes reports of three Chinese clinical studies and an Australian one of the use of cephalotaxus alkaloid drugs for the treatment of leukaemia. The doses of harringtonine used in the Chinese studies ranged from 0·15–0·3 mg kg^{-1} day^{-1} for five to 10 days,

repeated every seven to 14 days. The method of administration was not reported. Doses of homoharringtonine were 0·05–0·1 mg kg^{-1} administered using the same regimen. Mixtures of 1:3 and 2:1 harringtonine to homoharringtonine were also tested. The 1:3 mixture was administered in doses of 2 mg intramuscularly (im), and 4 mg intravenously (iv), and the 2:1 mixture was administered in doses of 4 mg iv. Both mixtures were given daily for an unspecified number of days. These dosages were reported safe, producing mild side effects, primarily hypotension, which reversed on stopping the drug.

Researchers in the U.S. (Coonley *et al.*, 1983; Warrell *et al.*, 1985) studied the possibility of administering homoharringtonine as a continuous infusion to avoid acute autonomic toxicity. Toxicity was characterized by dose-related and self-limited hypotension. Warrell *et al.* (1985) noted that a dose level up to 5 mg m^{-2} day^{-1} administered by continuous infusion for nine days was safe and effective for acute non-lymphoblastic leukaemia. Patients exhibited alopecia, diarrhoea, hyperglycaemia, and some weight gain in addition to hypotension. All side effects subsided after drug cessation.

MATERIALS AND METHODS

In vitro Studies

Several strains of *P. falciparum* have been maintained in our laboratory by the technique of continuous erythrocyte culture (Haynes *et al.*, 1976; Trager and Jensen, 1976), and FCR-3/Gambia, formerly chloroquine-sensitive, has become resistant. In contrast, Smith/Vietnam is chloroquine-resistant and Camp/Malay is pyrimethamine-resistant.

Homoharringtonine was dissolved in DMSO and RPMI 1640 media supplemented with 25 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid), 0·24% sodium bicarbonate and 10% heat-inactivated human serum. All cultures were flushed with 5% oxygen, 5% carbon dioxide and 90% nitrogen prior to incubation at 37°C.

Flask Cultures for Polyamine Analysis (Whaun and Brown, 1985)

Cultures set up for polyamine analysis in 25 mm^2 plastic culture flasks (Corning Glass Works, Corning, NY) were seeded at 0·12–0·15% parasitaemia. The drug, which contained under 1% final concentration of DMSO, was added to flask cultures of control or infected 6% red cell suspensions at daily media changes, after the first 24 hours. Vehicle-containing media were added at daily media changes to those cultures serving as drug controls. Cultures set up for polyamine analysis were terminated after three days. Perchloric acid extracts were prepared from infected red cells. Uninfected red cell cultures served as controls (Whaun and Brown, 1985). Analysis of extracts was carried out by an ultrasensitive automated method for the determination of polyamines by ion-pair high-performance liquid chromatography (Brown *et al.*, 1982).

Microtitre Well Studies for Assessing Parasite Growth (Desjardins *et al.*, 1979)

Microtitre plate (Costar, Cambridge, MA) cultures were set up at zero hour with drug or vehicle-containing media in 1% red cell suspensions with or without parasites. Those with parasites were seeded at 0·5%. The microtitre plates were placed in a humidified air-tight chamber (Division of Instrumentation, Walter Reed Army Institute of Research, Washington, DC), flushed with 5% oxygen, 5% carbon dioxide, 90% nitrogen, sealed, and incubated at 37°C for approximately 48 hours. Approximately 16–22 hours prior to the time of anticipated harvest, 0·5 μCi of [G-^{3}H]hypoxanthine (Amersham, Arlington Heights, IL, 1·2 Ci $mmol^{-1}$ specific activity) was added to each well. The contents of each well were harvested onto glass fibre filters (934-AH, Whatman Inc., Clifton, NJ) with a MASH II multiple automated sample harvester (Microbiological Associates, Bethesda, MD), dried, and

subsequently counted in scintillation vials containing 10 ml PCS (Amersham) in a Tracor Model 6882Z liquid scintillation counter (Tracor Analytic Inc., Elk Grove Village, IL).

Malarial growth in culture was assessed by determining the incorporation of [^{3}H] hypoxanthine into DNA and RNA (Desjardins *et al.*, 1979). ED_{50}, the dose required to cause 50% inhibition of parasite growth, was determined by least-squares non-linear regression.

In vivo Studies

Outbred Swiss ICR six- to seven-week-old mice were divided into 16 groups of seven mice each and inoculated with approximately 6×10^5 erythrocytes infected with *P. yoelii* parasites intraperitoneally (ip). Homoharringtonine was administered twice a day, either orally or subcutaneously (sc), in a volume of 10 ml of drug suspension per kg body weight on the third, fourth and fifth days after inoculation of parasites. The homoharringtonine was mixed in aqueous 0·5% hydroxyethyl cellulose—0·1% Tween 80, and ultra-sonicated when necessary. The doses were prepared using 100% free base of the drug. Groups 8 and 16 of the infected mice received the vehicle alone and served as negative controls.

Blood films and final group weights were taken on the sixth day following inoculation of parasites. Examination of Giemsa-stained blood films determined the per cent parasitaemia. The per cent suppression was calculated by comparing the average parasitaemia of the surviving mice with the parasitaemia of the negative controls. Toxicity was attributed to drug action when a 14% or greater weight change occurred, or when one or more mice died before blood films were taken. An effective dosage occurred when the drug produced a 90% suppression.

RESULTS

Effect on the Growth of *P. falciparum* in Culture

Homoharringtonine was effective against chloroquine-resistant strains, at low concentrations. A comparison of the ED_{50}, the dose associated with 50% inhibition of parasite growth, of homoharringtonine and chloroquine for three strains is listed in Table 1. As shown, Smith/Vietnam, a well-established chloroquine-resistant strain (ED_{50} chloroquine 219 nM) had an ED_{50} of 2·7 nM; FCR-3/Gambia, a slightly chloroquine-resistant strain (ED_{50} chloroquine 32 nM), 3·4 nM; and Camp/Malay, a chloroquine-sensitive strain (ED_{50} chloroquine 19 nM), 2·5 nM for homoharringtonine. Morphological changes in blood films of *P. falciparum*-infected red cell cultures exposed to homoharringtonine showed nuclear pyknosis, arrest of parasite maturation beyond ring forms, and tortuous rings.

TABLE 1

ED_{50}, the dose of homoharringtonine and chloroquine required to cause 50% inhibition of growth of three strains of Plasmodium falciparum

	In vitro *suppression of* Plasmodium falciparum *treated with homoharringtonine or chloroquine*	
Strain	*ED_{50} Homoharringtonine*	*ED_{50} Chloroquine*
Smith/Vietnam	1·48 ± 0·02 ng ml^{-1} (2·7 nM)	70·0 ng ml^{-1} (219 nm)
Camp/Malay	1·36 ± 0·02 ng ml^{-1} (2·5 nM)	6·12 ng ml^{-1} (19 nM)
FCR-3/Gambia	1·88 ± 0·02 ng ml^{-1} (3·4 nM)	10·25 ng ml^{-1} (32 nM)

TABLE 2

Polyamine metabolism of Plasmodium falciparum-*infected red cell cultures. Identical superscript letters refer to* P *tests of significance of two compared groups*

	Polyamine levels in cell cultures		
	Putrescine	*Spermidine*	*Spermine*
(A) CELL PELLETS (mean ± S.D. nmol 10^{-10} RBC)			
Red cells (RBC) alone	$10{\cdot}3 \pm 0{\cdot}4^{a}$	$56{\cdot}6 \pm 2{\cdot}4^{c}$	$16{\cdot}0 \pm 0{\cdot}7^{e}$
RBC + homoharringtonine	$9{\cdot}4 \pm 0{\cdot}2^{a}$	$40{\cdot}2 \pm 2{\cdot}3^{c}$	$22{\cdot}3 \pm 1{\cdot}9^{e}$
Parasitized RBC alone	$10{\cdot}3 \pm 0{\cdot}6^{b}$	$44{\cdot}6 \pm 2{\cdot}2^{d}$	$8{\cdot}0 \pm 0{\cdot}2^{f}$
Parasitized RBC + homoharringtonine	$9{\cdot}1 \pm 0{\cdot}3^{b}$	$41{\cdot}0 \pm 1{\cdot}1^{d}$	$11{\cdot}3 \pm 1{\cdot}1^{f}$
	Putrescine	*Spermidine*	*Spermine*
(B) EXTRACELLULAR FLUID (mean ± S.D. pmol ml^{-1})			
RBC alone	$178{\cdot}2 \pm 7{\cdot}5^{g}$	$124{\cdot}4 \pm 8{\cdot}2^{i}$	$42{\cdot}8 \pm 1{\cdot}5^{k}$
RBC + homoharringtonine	$177{\cdot}2 \pm 3{\cdot}4^{g}$	$101{\cdot}9 \pm 4{\cdot}1^{i}$	$57{\cdot}4 \pm 3{\cdot}5^{k}$
Parasitized RBC	$252{\cdot}0 \pm 12{\cdot}2^{h}$	$109{\cdot}8 \pm 2{\cdot}6^{j}$	$62{\cdot}0 \pm 3{\cdot}8^{l}$
Parasitized RBC + homoharringtonine	$254{\cdot}8 \pm 3{\cdot}0^{h}$	$111{\cdot}7 \pm 3{\cdot}2^{j}$	$121{\cdot}0 \pm 7{\cdot}8^{l}$

[a] *P* under 0·025.
[b] *P* under 0·05.
[c] *P* under 0·005.
[d] *P* under 0·025.
[e] *P* under 0·01.
[f] *P* under 0·005.
[g] *P* N.S.
[h] *P* N.S.
[i] *P* under 0·025.
[j] *P* N.S.
[k] *P* under 0·005.
[l] *P* under 0·005.

TABLE 3

In vivo *administration of homoharringtonine subcutaneously to* Plasmodium yoelii-*infected mice. See text for further details*

	In vivo *suppression of* Plasmodium yoelii *in mice when treated subcutaneously with homoharringtonine*					
Group	*Daily dose (mg kg^{-1})*	*Total dosage*	*% Parasitaemia of each mouse*	*Total no. survivors*	*Average parasitaemia*	*% Suppression*
1	64·0	192·0	—, —, —, —, —, —, —,	0	N.D.	N.D.
2	32·0	96·0	—, —, —, —, —, —, —,	0	N.D.	N.D.
3	16·0	48·0	—, —, —, —, —, —, —,	0	N.D.	N.D.
4	8·0	24·0	0·15, 1·0, 0·05, 1·0, 0·25, —, —,	5	0·49	98·85
5	4·0	12·0	3·0, 13·0, 4·0, 0·02, 1·0, 0·1, 5·0	7	3·73	91·21
6	2·0	6·0	16, 40, 20, 13, 5, 28, 23	7	20·71	51·18
7	1·0	3·0	33, 42, 65, 31, 23, 25, 35	7	36·29	14·48
8	Negative control		59, 42, 39, 58, 40, 30, 29	7	42·43	

90% Suppression *v.* daily dose = 3·8 mg kg^{-1}.
— Mouse died toxic death before day 6.
N.D. Not determined because of toxic death.

Effect on Polyamine Metabolism

Although putrescine and spermidine decreased in the drug-exposed cell pellets, spermine levels rose (Table 2). In contrast, in the supernatant extracts, only spermine levels rose in the drug-treated groups, with the parasitized cells showing the larger increase.

Effect of *in vivo* Treatment of *Plasmodium yoelii*-infected Mice

The results of the *in vivo* tests are shown in Tables 3 and 4 and in Figs 2 and 3 for subcutaneous

TABLE 4

In vivo *administration of homoharringtonine orally to* Plasmodium yoelii-*infected mice*

	In vivo *suppression of* Plasmodium yoelii *in mice when treated orally with homoharringtonine*					
Group	*Daily dose (mg kg^{-1})*	*Total dosage*	*% Parasitaemia of each mouse*	*Total no. survivors*	*Average parasitaemia*	*% Suppression*
9	32·0	96·0	—, —, – , —, —, —, —,	0	N.D.	N.D.
10	16·0	48·0	—, —, —, —, —, —, —,	0	N.D.	N.D.
11	8·0	24·0	0·02, 0·01, 0·01, —, —, —, —,	3	0·01	99·97
12	4·0	12·0	0·05, 1·0, 0·25, 0·5, 6·0, 0·25, 0·5	7	1·22	97·55
13	2·0	6·0	8, 12, 14, 5, 22, 16, 12	7	12·71	74·50
14	1·0	3·0	13, 10, 15, 35, 31, 24, 7	7	19·29	61·32
15	0·5	1·5	44, 21, 16, 39, 29, 27, 56	7	33·14	33·52
16	Negative control		35, 29, 66, 71, 45, 50, 53	7	49·86	

90% Suppression *v.* daily dose = 2·8 mg kg^{-1}.
— Signifies mouse died toxic death before day 6.
N.D. signifies not determined because of toxic death.

and oral treatments respectively. The effective dosage for homoharringtonine, administered under the conditions outlined above, is 3·8 mg kg^{-1} day^{-1} sc and 2·8 mg kg^{-1} day^{-1} orally.

DISCUSSION

Homoharringtonine has been administered both *in vitro* and *in vivo* to systems infected with malaria. Both tests showed significant antimalarial activity. The data in Table 1 showed homoharringtonine to be from four to 47 times more effective against *P. falciparum*-infected human red cells than the standard antimalarial drug, chloroquine.

Pathologically in L1210 tumour cells, harringtonine has been demonstrated to be associated with nuclear karyorrhexis (Chou *et al.*, 1983). Similar changes in parasite-infected human red cells exposed to homoharringtonine have been noted. Other investigators (Huang, 1975) have reported that harringtonine (and probably homoharringtonine) acts by inhibiting protein and nucleic acid synthesis of both DNA and RNA in tumour cells. It is thought to block peptide bond formation and aminoacyl-tRNA binding (Fresno *et al.*, 1977). It is somewhat cell-cycle specific, with the most marked action in the G_1 and G_2 phase. These observations

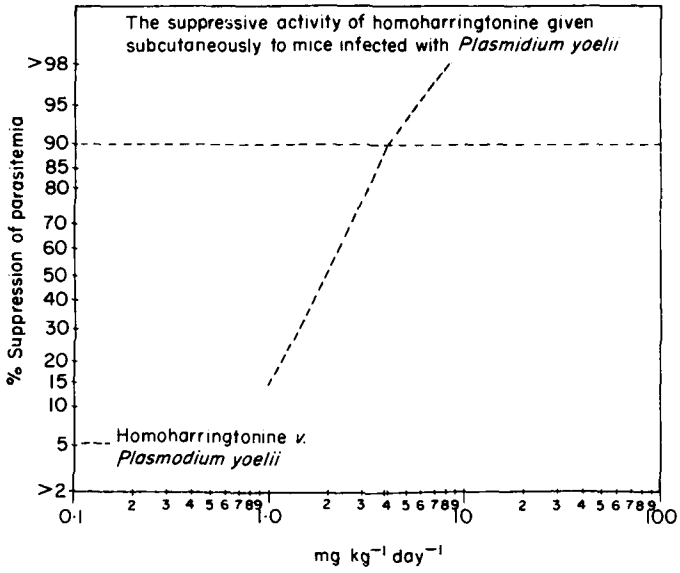


Fig. 2. *In vivo* tests of subcutaneous administration of homoharringtonine in the treatment of *Plasmodium yoelii*-infected mice.

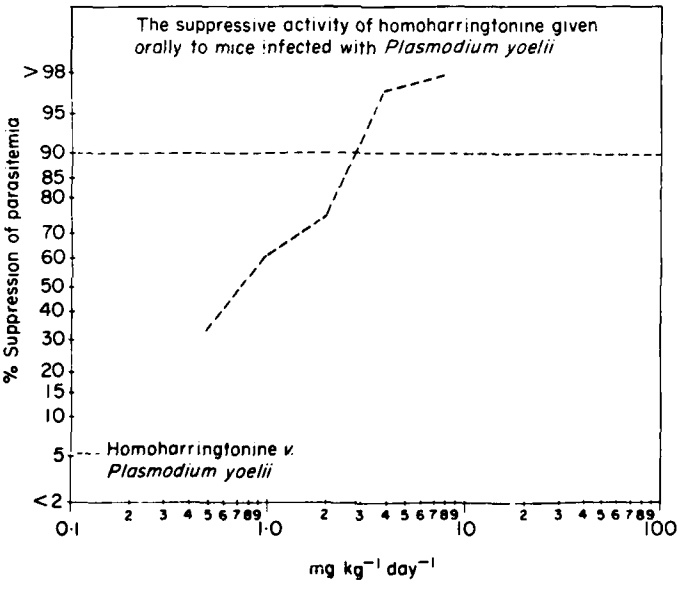


Fig. 3. *In vivo* tests of the oral administration of homoharringtonine in the treatment of *Plasmodium yoelii*-infected mice.

were made in monolayer cell cultures of synchronized human oral epidermoid carcinoma cells and human cervical carcinoma cells, as well as murine fibroblast cells (Baaske and Heinstein, 1977).

Our findings on polyamine metabolite levels suggest that decreased putrescine levels in cell pellets may be associated with growth inhibition. This might be accompanied by decreased spermidine levels because of decreased putrescine availability for spermidine synthesis. Decreased utilization, or perhaps a block in utilization of spermine could result in a build-up of spermine, noted in our studies. These results are consistent with a non-specific effect of this drug on polyamine metabolism. Further studies are needed to understand the mechanism of the effect on polyamine levels.

The mice studies suggest that a daily dosage between 2·8 mg kg^{-1} and 3·8 mg kg^{-1} would be effective. The clinical cancer studies showed that safe dosages of 5·0 mg m^{-2} administered by continuous infusion were effective. By comparison, the mice dosages are very high. However, differences in host species, pathogenic organism, and mode of administration render dosage comparisons misleading and unrewarding.

Homoharringtonine has been found to be extremely effective in halting growth of *P. falciparum* in human red cells. With the current interest in homoharringtonine as an antitumour agent, safety and dosage formulation concerns are currently being worked out in human Phase II clinical trials at the National Cancer Institute. The increased effectiveness of homoharringtonine for chloroquine-resistant malaria makes this drug an attractive addition to the current armamentarium for the treatment of selected patients with drug-resistant malaria.

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